23,24,25-Trihydroxyvitamin D_3, 24,25,26-Trihydroxyvitamin D_3, 24-Keto-25-hydroxyvitamin D_3, and 23-Dehydro-25-hydroxyvitamin D_3: New in Vivo Metabolites of Vitamin D_3†

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ABSTRACT: Four new in vivo metabolites of vitamin D_3 were isolated from the blood plasma of chicks given large doses of vitamin D_3. The metabolites were isolated by methanol–chloroform extraction and a series of chromatographic procedures. By use of mass spectrometry, ultraviolet absorption spectrophotometry, and specific chemical reactions, the metabolites were identified as 23,24,25-trihydroxyvitamin D_3, 24,25,26-trihydroxyvitamin D_3, 24-keto-25-hydroxyvitamin D_3 and 23-dehydro-25-hydroxyvitamin D_3.

Vitamin D_3 is known to undergo a series of metabolic modifications in vertebrates (DeLuca, 1980; Kodicek, 1974; DeLuca & Schnoes, 1976). The vitamin is hydroxylated in liver to form 25-hydroxyvitamin D_3 (25-OH-D_3).[1] In kidney, 25-OH-D_3 can be further metabolized to yield 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$], 25,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$], 23,25-dihydroxyvitamin D_3 [23,25-$(OH)_2D_3$], 25-hydroxyvitamin D_3 26,23-lactone (25-OH-D_3-26,23-lactone), or 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], the active form of the vitamin. 1,25-$(OH)_2D_3$ is metabolized further to either 1,24(*R*),25-trihydroxyvitamin D_3 [1,24(*R*),25-$(OH)_3D_3$] or to a 23-acid called calcitroic acid (Esvelt et al., 1979).

Previously, 25-OH-D_3-26,23-lactone (Wichmann et al., 1979) and 24(*R*)-hydroxyvitamin D_3 [24(*R*)-OH-D_3] (Wichmann et al., 1981) were isolated and identified from the blood plasma of chicks given large doses of vitamin D_3. We report here the isolation and identification of four new in vivo metabolites of vitamin D_3 from the plasma of chicks receiving high doses of vitamin D_3. These metabolites are 24-keto-25-hydroxyvitamin D_3 (24-keto-25-OH-D_3), previously identified from in vitro incubation of kidney homogenates with 25-OH-D_3 (Takasaki et al., 1981), 23-dehydro-25-hydroxyvitamin D_3 (23-dehydro-25-OH-D_3), 23,24,25-trihydroxyvitamin D_3 [23,24,25-$(OH)_3D_3$], and 24,25,26-trihydroxyvitamin D_3 [24,25,26-$(OH)_3D_3$].

Materials and Methods

Plasma Procurement and Extraction. Sixty 12-week-old, white Leghorn cockerels (Northern Hatcheries, Beaver Dam, WI) raised on a commercial diet (Ralston Purina, St. Louis, MO) were each dosed intramuscularly with 10^5 IU of vitamin D_3 (Aldrich Chemicals, Milwaukee, WI) in 50 μL of ethanol daily for 3 days. The vitamin purity was found to be >99.9% by high-performance liquid chromatography (HPLC). On the fourth day, each chick was dosed intramuscularly with a total of 1.5×10^7 IU of vitamin D_3. Four days after this dose, blood was collected by cardiac puncture, separated, and extracted as previously described (Wichmann et al., 1979, 1981).

Chromatographic Purification of Compounds I and II. The extract from 1160 mL of plasma was chromatographed on a 3 × 30 cm Sephadex LH-20 column eluted with hexane–methanol–chloroform (9:1:1). Eighty 23-mL fractions were collected. The presence of vitamin D metabolites was detected by using the competitive protein binding assay described by Shepard et al. (1979). The binding peak, eluting in the 25-OH-D_3 region (fractions 11–19; 254–437 mL), was collected. The sample was then chromatographed on a 2 × 57 cm Lipidex 5000 column eluted with hexane–chloroform (92:8). Sixty 16-mL fractions were collected and assayed as above. The fractions in the 25-OH-D_3 region (fractions 37–45; 592–720 mL) were pooled, and the solvent was removed by evaporation under vacuum.

The sample was further purified by HPLC using a Waters Model ACP/GPC 204 instrument (Waters Associates, Inc., Milford, MA) equipped with a Model 450 variable-wavelength detector and wavelength drive attachment. The Lipidex fraction was chromatographed on a 0.45 × 25 cm microparticulate silica column (Zorbax-SIL, Du Pont, Wilmington, DE) eluted with 2% 2-propanol in hexane. On this system standard 25-OH-D_3 elutes at 36 mL. The material eluting at 32–34 mL was pooled for isolation of compound I, and the 34–36-mL fractions were pooled for isolation of compound II. Both fractions were chromatographed separately by HPLC on 0.45 × 25 cm octadecylsilane bonded to silica column (Zorbax ODS, Du Pont) eluted with 15% water in methanol. 25-OH-D_3 elutes at 28 mL on this system. Compound I was collected, eluting at 24–28 mL, and compound II was collected at 12–13 mL. Both compounds exhibited a UV absorption at λ_{max} = 265 nm.

Final purification of both compounds was performed on HPLC using a 0.45 × 25 cm microparticulate silica column eluted with 4% 2-propanol in hexane. Standard 25-OH-D_3 eluted at 15 mL on this system. Compound I was eluted at 14–15 mL while compound II eluted at 16–17 mL.

Chromatographic Purification of Compounds III and IV. Chloroform extract from 380 mL of plasma was chromato-

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[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 23,25-$(OH)_2D_3$, 23,25-dihydroxyvitamin D_3; 25-OH-D_3-26,23-lactone, 25-hydroxyvitamin D_3 26,23-lactone; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,24(*R*),25-$(OH)_3D_3$, 1,24(*R*),25-trihydroxyvitamin D_3; 24(*R*)-OH-D_3, 24(*R*)-hydroxyvitamin D_3; 24-keto-25-OH-D_3, 24-keto-25-hydroxyvitamin D_3; 23-dehydro-25-OH-D_3, 23-dehydro-25-hydroxyvitamin D_3; 23,24,25-$(OH)_3D_3$, 23,24,25-trihydroxyvitamin D_3; 24,25,26-$(OH)_3D_3$, 24,25,26-trihydroxyvitamin D_3; HPLC, high-performance liquid chromatography; BSTFA, *N,O*-bis-(trimethylsilyl)trifluoroacetamide.

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graphed on a 3 × 30 cm Sephadex LH-20 column eluted with hexane–chloroform–methanol (9:1:1). Fractions were collected and assayed as above. The vitamin D metabolites eluting from 1150 to 1472 mL were collected and prepared for HPLC.

Primary HPLC purification was performed on a 0.45 × 25 cm microparticulate silica column (Zorbax-SIL, Du Pont) eluted with 12% 2-propanol in hexane. Standard 1,25-$(OH)_2D_3$ elutes at 18 mL on this system. The material eluting at 10–12 mL was collected for the isolation of compound III, while the material eluting at 17–22 mL was collected for the isolation of compound IV. The compounds were then separately subjected to HPLC on a 0.45 × 25 cm ODS column eluted with 20% water in methanol. Standard 1,25-$(OH)_2D_3$ eluted at 25 mL on this system. Compound III was eluted at 7–8 mL and compound IV eluted at 9–11 mL. Both compounds III and IV had UV absorption at λ_{max} = 265 nm.

Final purification of both compounds III and IV was performed on a 0.45 × 25 cm microparticulate silica column eluted with 14% 2-propanol in hexane. Compound III was eluted at 7.5–9.5 mL and compound IV eluted at 14.5–18 mL.

Chemical Modification of Metabolites. (1) $NaBH_4$ Reduction. Ten microliters of a 5% methanolic solution of $NaBH_4$ was added to the sample in 15 μL of methanol. The reaction was allowed to proceed for 30 min at 25 °C at which time 200 μL of water was added and the product extracted 3 times with 200 μL of CH_2Cl_2. The CH_2Cl_2 was removed under N_2 and the sample used for further analysis.

(2) Periodate Cleavage. To the sample in 15 μL of methanol was added 10 μL of 5% aqueous (pH 4.5) $NaIO_4$. After 1 h at 25 °C, 100 μL of H_2O was added and the product extracted 3 times with 200 μL of CH_2Cl_2. The CH_2Cl_2 was removed under N_2.

(3) Trimethylsilylation. Metabolites III and IV were silylated by treating a sample in 15 μL of pyridine with 10 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylsilyl chloride (Me_3SiCl). After 40 min at 35 °C, reagents were removed under N_2 and the sample was dissolved in hexane for analysis. These conditions gave incomplete silylation and resulted in tri- and tetramethylsilyl product mixtures.

The quantitative trimethylsilylation of metabolites I and II was achieved by reacting a sample in 20 μL of pyridine with 25 μL of BSTFA containing 1% Me_3SiCl. After 45 min at 55 °C, reagents were removed under N_2 and the sample was dissolved in hexane for analysis.

(4) Ozonolysis. To the sample in 10 μL of CH_2Cl_2 at −78 °C was added 100 μL of a CH_2Cl_2 solution saturated with O_3 at −10 °C. After 2 min at −78 °C, 15 μg of triphenylphosphine in 10 μL of CH_2Cl_2 was added. The solution was allowed to reach 25 °C and the solvents were concentrated to 5 μL under N_2.

Chromatography of Reaction Products. (1) Compound I. A total of 450 ng was treated with $NaBH_4$ and the crude product quantitatively silylated. The silyl ether product was purified on a 0.46 × 25 cm microparticulate silica column eluted with 0.2% ethyl acetate in hexane. The major 254-nm absorbing material eluting at 34–36 mL was collected and used for mass spectrometry.

Compound I (100 ng) was subjected to ozonolysis. The concentrated products were analyzed by chemical ionization GC–MS on a 2 mm × 2 m 3% OV-1 column using methane as the carrier/reagent gas at a flow rate of 30 mL/min. The analysis was temperature programmed from 100 to 220 °C at 5 °C/min. The major sample peak eluted at 13.75 min at a temperature of 169 °C.

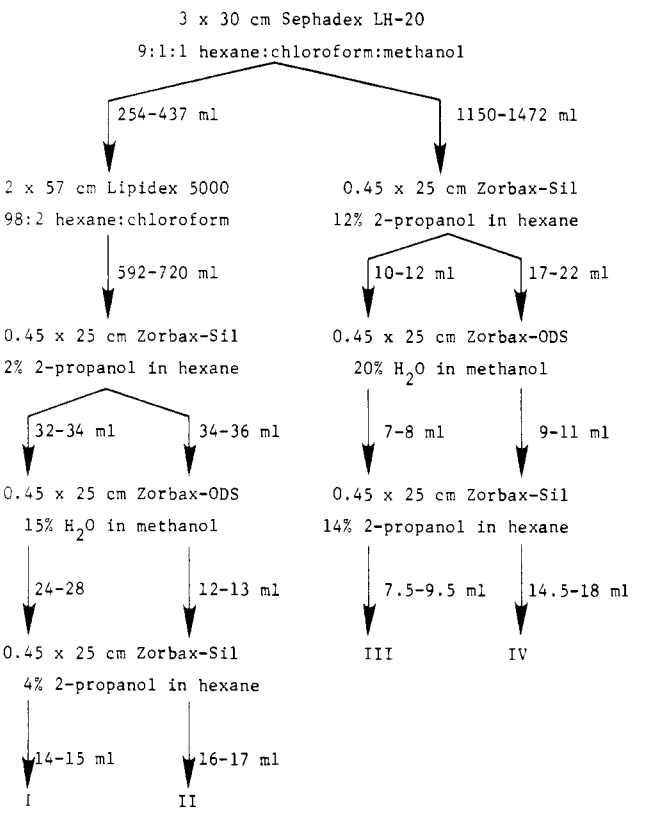

FIGURE 1: Flow sheet of the isolation of compounds I–IV.

(2) Compound II. A total of 400 ng was quantitatively silylated and used directly for mass spectrometry.

A total of 700 ng was reduced with $NaBH_4$ and chromatographed by HPLC on a 0.46 × 25 cm microparticulate silica column eluted with 6% 2-propanol in hexane. The major 265-nm absorbing peak eluting at 20–24 mL was collected. The product (500 ng) was quantitatively silylated and used directly for mass spectrometry. A total of 50 ng of product was used for cochromatography with standard 24,25-$(OH)_2D_3$ on the 6% 2-propanol system above. A total of 150 ng of product was subjected to periodate cleavage and HPLC analysis on the 6% 2-propanol system above. Standard 24,25-$(OH)_2D_3$ (200 ng) was subjected to identical periodate cleavage and used for comparison with the periodate cleavage product of compound II.

(3) Compound III. The entire purified sample (350 ng) was partially silylated and used directly for mass spectrometry.

(4) Compound IV. A total of 400 ng (half of the purified metabolite) was subjected to partial silylation and used directly for mass spectrometry.

Mass Spectrometry. All mass spectra except the ozonolysis product of compound I were obtained with an AEI Model MS9 mass spectrometer interfaced with a DS-50 data system (Kratos, Westwood, NJ). All spectra were run at 70 eV with a source temperature between 90 and 140 °C above ambient.

The ozonolysis product of compound I was analyzed on a Finnigan 4000 EI/CI GC-MS (Finnigan Corp., Sunnyvale, CA) interfaced with INCOS data system (Finnigan Corp.). Methane was added as the makeup gas to give a source pressure of 0.13 torr.

Results

A flow sheet outlining the chromatographic procedures used for purification of the metabolites is presented in Figure 1. By use of a stop-flow wavelength scan on the final HPLC column for each metabolite, the characteristic UV absorption with λ_{max} = 265 nm of the intact vitamin D_3-*cis*-triene chro-

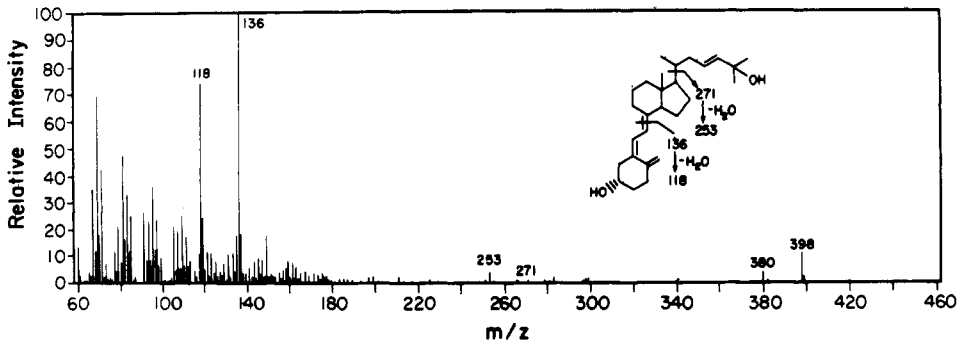

FIGURE 2: Mass spectrum of 23-dehydro-25-OH-D_3.

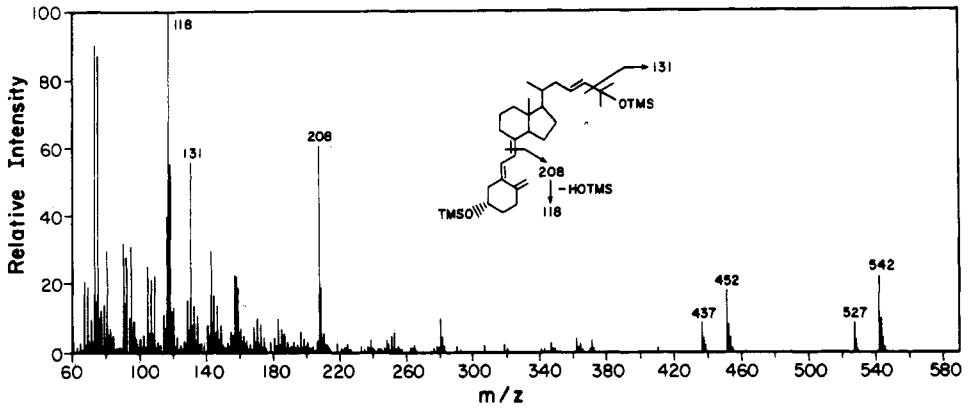

FIGURE 3: Mass spectrum of the $(Me_3Si)_2$ derivative of 23-dehydro-25-OH-D_3.

mophore could be demonstrated for all compounds. By comparison with peak areas of known amounts of standard 25-OH-D_3 or 1,25-$(OH)_2D_3$, the total amounts of isolated metabolites were as follows: compound I, 850 ng; compound II, 1.7 μg; compound III, 400 ng; compound IV, 900 ng. Without recovery corrections, the plasma levels of each compound were 0.7 ng/mL for compound I, 1.5 ng/mL for compound II, 1.1 ng/mL for compound III, and 2.4 ng/mL for compound IV.

The mass spectrum of compound I is presented in Figure 2. Major ions, relative intensities, and structural assignments are as follows: m/z 398, 11, M^+; 380, 5, $M^+ - H_2O$; 271, 2, M^+ – side chain; 253, 5, 271 – H_2O; 136, 100, (A ring + C_6 + C_7)$^+$; 118, 74, 136 – H_2O. The apparent molecular ion at m/z 398 is consistent with the incorporation of an oxygen atom and an unsaturation in the vitamin molecule. Characteristic vitamin D_3 cleavage ions at m/z 271, 253, 136, and 118 show that the metabolic alteration of the vitamin has occurred exclusively on the side chain and that the secosteroid nucleus is unaltered. Likely side-chain modifications consistent with these data include (a) the formation of a carbonyl functionality, (b) the formation of a cyclic ether (e.g., epoxide), and (c) the presence of a hydroxy group plus double bond. Treatment of the metabolite with borohydride and subsequent silylation gave a product whose mass spectrum is shown in Figure 3. Major ions, relative intensities, and structural assignments of this mass spectrum are as follows: m/z 542, 22, M^+; 527, 8, $M^+ - CH_3$; 452, 18, $M^+ - HOSiMe_3$; 437, 8, 452 – CH_3; 208, 60, (A ring + C_6 + C_7)$^+$; 131, 56, $C_3H_6OSiMe_3^+$; 118, 100, 208 – $HOSiMe_3$. The apparent molecular ion at m/z 542 indicates formation of a bis(trimethylsilyl) ether derivative of a diol of molecular weight 398 (i.e., the molecular weight of the original metabolite). This result eliminates ketone and cyclic ether structures and suggests an unsaturated monohydroxylated side chain. The C-25 position of the hydroxy group can be deduced from the prominent ion at m/z 131 (see Figure 3), whereas the ions at 208 (A ring$^+$ + C_6 + C_7) and 118 are characteristic fragments

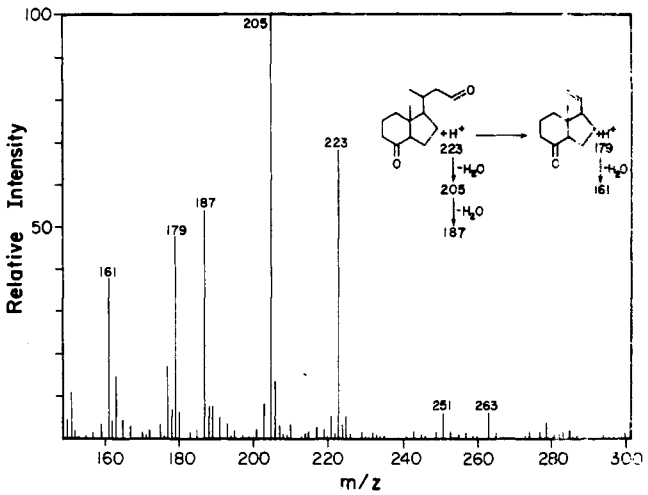

FIGURE 4: Methane chemical ionization mass spectrum of the ozonolysis product from 23-dehydro-25-OH-D_3.

of vitamin D-3β-$OSiMe_3$ derivatives and reconfirm an intact A-ring moiety. The presence and the position of the side-chain double bond was established by ozonolysis of compound I and methane CI GC–MS of the ozonolysis product. The CI mass spectrum of this product is shown in Figure 4. The apparent protonated molecular ion $(M + H)^+$ at m/z 223, accompanied by the low-intensity adduct ions of m/z 251 $(M + C_2H_5)^+$ and 263 $(M + C_6H_5)^+$, indicates a molecular weight of 222 for this ozonolysis product as required for the keto-aldehyde resulting from cleavage of the C-7–C-8 and a C-23–C-24 bond in the metabolite (see the structure in Figure 4). The structure of this ozonolysis fragment is confirmed by the successive loss of two H_2O molecules from the protonated molecular ion to give the peaks at m/z 205 and 187 and the presence of the McLaferty rearrangement ion at m/z 179 [loss of C_2H_4O from $(M + H)^+$] followed by loss of H_2O to yield the ion at m/z 161. Thus, the unsaturation in the side chain must be in the C-23,C-24 position, and the structure of compound I is 23-

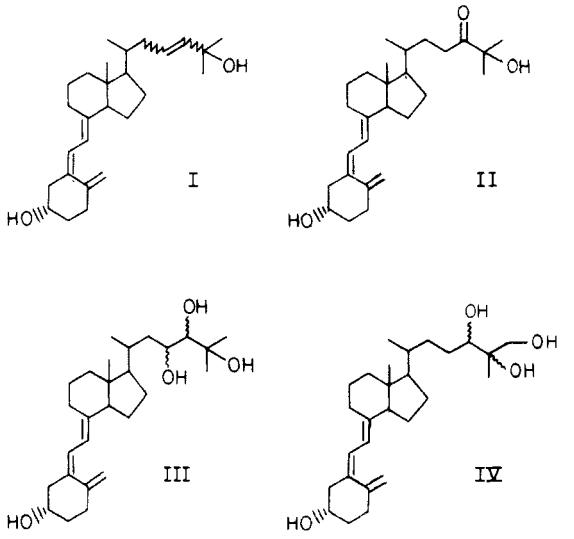

FIGURE 5: Structures of 23-dehydro-25-OH-D_3 (I), 24-keto-25-OH-D_3 (II), 23,24,25-$(OH)_3D_3$ (III), and 24,25,26-$(OH)_3D_3$ (IV).

dehydro-25-hydroxyvitamin D_3 (Figure 5, I). The stereochemistry of the side-chain double bond cannot be determined from our data.

The mass spectrum of compound II is presented in Figure 6. Major ions, relative intensities, and structural assignments are as follows: m/z 414, 18, M^+; 396, 1, $M^+ - H_2O$; 381, 2, $M^+ - H_2O - CH_3$; 271, 4, M^+ – side chain; 253, 5, 271 – H_2O; 136, 100, (A ring + C_6 + C_7)$^+$; 118, 72, 136 – H_2O. The apparent molecular ion at m/z 414 suggests the incorporation of two oxygens and one degree of unsaturation in the vitamin molecule. Characteristic fragment ions of the vitamin at m/z 271, 253, 136, and 118 show that all metabolic alteration has occurred on the side chain.

For determination of the nature of the oxygen and unsaturation functionalities, 500 ng of compound II was quantitatively silylated and used for mass spectrometry. Major ions, relative intensities, and structural assignments for this spectrum (not shown) are as follows: m/z 558, 5, M^+; 468, 3, M^+ – $HOSiMe_3$; 208, 10, (A ring + C_6 + C_7)$^+$; 131, 98, $C_3H_6OSiMe_3^+$; 118, 100, 208 – $HOSiMe_3$. The apparent molecular ion at m/z 558 indicates the formation of a bis(trimethylsilyl) ether derivative. Thus, one side-chain oxygen is present as a free hydroxyl group, and the other oxygen must be either an ether or a carbonyl in order to satisfy the requirement of an unsaturation in the molecule. The free hydroxyl group can be assigned to C-25 on the basis of the intense peak at m/z 131 which arises from cleavage between carbons 24 and 25 and formation of the $C_3H_6OSiMe_3^+$ ion.

Treatment of 700 ng of compound II with $NaBH_4$ gave a major reduction product with chromatographic properties on HPLC very similar to those of standard 24,25-$(OH)_2D_3$. Conclusive proof that the reduction product was a 24,25-$(OH)_2D_3$ (probably a mixture of the 24*R* and 24*S* epimers) was provided by the mass spectrum of the silylated derivative, by comigration with authentic 24,25-$(OH)_2D_3$ on HPLC, and by periodate cleavage and HPLC analysis of the resulting aldehyde product.

The mass spectrum of the Me_3Si derivative of the reduction product (not shown) exhibited the following pattern: m/z 632, 10, M^+; 617, 5, $M^+ - CH_3$; 542, 6, $M^+ - HOSiMe_3$; 527, 4, 542 – CH_3; 501, 3, $M^+ - C_3H_6OSiMe_3$; 411, 3, 501 – $HOSiMe_3$; 343, 2, M^+ – side chain; 253, 6, 343 – $HOSiMe_3$; 208, 51, (A ring + C_6 + C_7)$^+$; 131, 100, $C_3H_6OSiMe_3^+$; 118, 67, 208 – $HOSiMe_3$. This spectrum is identical with that of the tris(trimethylsilyl) ether derivative of 24,25-$(OH)_2D_3$.

The comigration on HPLC of 50 ng of the reduction products of compound II and 25 ng each of the 24*R* and 24*S* epimers of 24,25-$(OH)_2D_3$ is shown in Figure 7A. This chromatographic system would not resolve the C-24 epimers, and the single UV-absorbing peak in the chromatograph therefore indicates the reduction product to be 24,25-$(OH)_2D_3$. Arrows indicate the elution position of various other side-chain dihydroxylated vitamin D_3 derivatives on this system.

The HPLC chromatogram of the periodate cleavage product of reduced compound II is shown in Figure 7B. The arrow indicates the elution position of the 24-aldehyde produced by periodate cleavage of standard 24,25-$(OH)_2D_3$.

Identical elution positions for the periodate cleavage product in Figure 7B, cochromatography in Figure 7A, and identical mass spectra of the $(Me_3Si)_3$ derivatives of reduced compound II and standard 24,25-$(OH)_2D_3$ prove that compound II is reduced by $NaBH_4$ to 24,25-$(OH)_2D_3$. Thus, compound II must be 3β,25-dihydroxy-24-oxo-9,10-seco-5,7,10(19)-cholestatriene or 24-keto-25-hydroxyvitamin D_3 (Figure 5, II).

Because of the small amount of compound III available for analysis, the entire sample was silylated and used directly for mass spectrometry. Although the reaction conditions chosen resulted in incomplete silylation to a mixture of the tri- and tetrasilyl derivatives, the mass spectrum of the product, shown in Figure 8, is nevertheless readily and unambiguously interpretable. Major ions, relative intensities, and structural assignments are as follows: m/z 720, 2.5, M^+ [$(Me_3Si)_4$ derivative]; 648, 10, M^+ [$(Me_3Si)_3$ derivative]; 633, 0.5, 648 – CH_3; 558, 0.7, 648 – $HOSiMe_3$; 543, 0.9, 558 – CH_3; 487, 5.2, 720 – $C_4H_7(OSiMe_3)_2$ and 648 – $C_4H_7OHOSiMe_3$; 397, 2, 487 – $HOSiMe_3$; 307, 5, 397 – $HOSiMe_3$; 208, 48, (A ring + C_6 + C_7)$^+$; 131, 80, $C_3H_6OSiMe_3$; 118, 100, 208 – $HOSiMe_3$.

The molecular ion at m/z 720 is consistent with a tetrakis(trimethylsilyl) ether derivative of a trihydroxylated vitamin D_3 metabolite. That compound III is a trihydroxyvitamin D_3

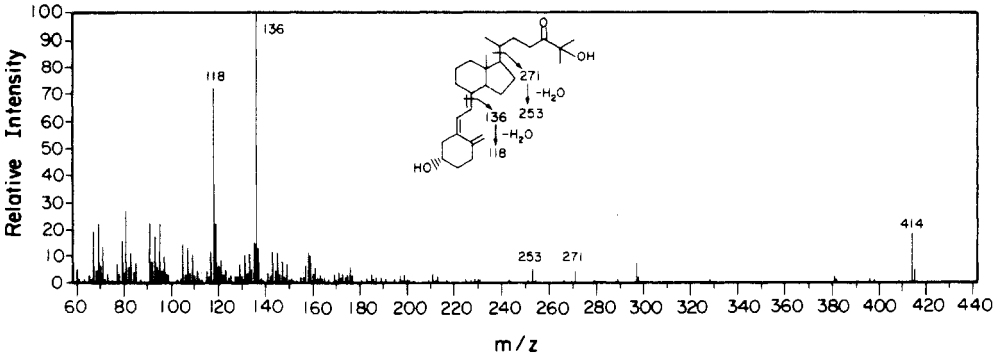

FIGURE 6: Mass spectrum of 24-keto-25-OH-D_3.

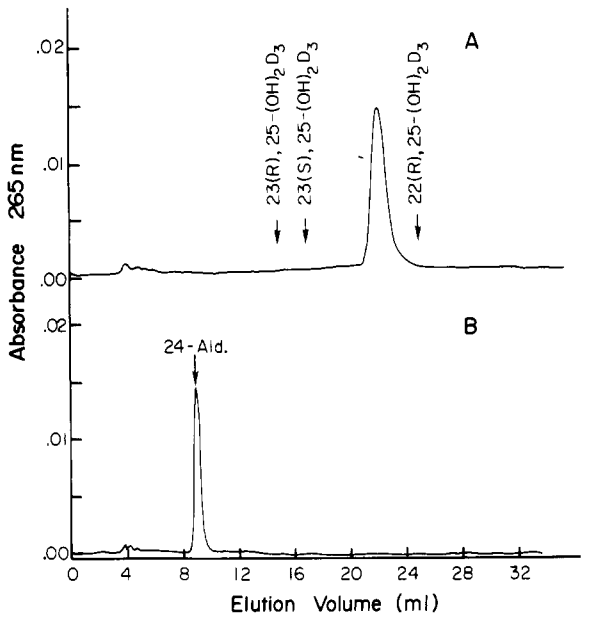

FIGURE 7: (A) HPLC chromatogram of 50 ng of the $NaBH_4$ reduction product of 24-keto-25-OH-D_3 and 25 ng of each 24 epimer of 24,25-$(OH)_2D_3$. Arrows indicate elution positions of other dihydroxylated vitamin D_3 compounds. (B) HPLC chromatogram of 50 ng of the periodate cleavage product of reduced 24-keto-25-OH-D_3. The arrow indicates the elution position of the 24-aldehyde obtained from periodate cleavage of 24,25-$(OH)_2D_3$.

derivative is also indicated by the ion at m/z 648. This ion is expected from a partially trimethylsilylated, trihydroxylated vitamin D_3 metabolite with one underivatized hydroxyl functionality. The (A ring + C_6 + C_7) fragment ions at m/z 208 and 118 confirm an intact seco-steroid nucleus and the 3β-hydroxyl group as the only functionality in ring A. The ion at m/z 487 shows that all three additional hydroxyl groups are on the side chain and further establishes the location of one of these at C-23. For the case of the trisilylated derivative of 23,25-$(OH)_2D_3$, it has been shown previously that of the four ions possible by 23-$OSiMe_3$-directed fragmentation only the ion at m/z 487 is present (Tanaka et al., 1981). This ion results from cleavage between C-23 and C-24 with loss of the four-carbon side-chain fragment (see Figure 8). Thus, compound III must be 23-hydroxylated, and the other two hydroxyl groups must be on carbons 24–27. The position of one of these hydroxyls is apparent from the intense peak at m/z 131. This ion is formed from C-25 $OSiMe_3$ directed fragmentation between carbons 24 and 25 (Figure 8) and rules out further hydroxylation of either carbons 26 or 27. The third hydroxyl group, therefore, must be on carbon 24. The only structure consistent with the mass spectrum is 23,24,25-$(OH)_3D_3$ (Figure 5, III).

The structure of compound IV (0.9 μg) was determined from the mass spectra of the metabolite and its silylated derivative.

The mass spectrum of compound IV, shown in Figure 9, exhibits a typical vitamin D pattern with peaks at m/z 432, 12%, M^+; 399, 3%, $M^+ - H_2O - CH_3$; 136, 100%, (A ring + $C_6 + C_7)^+$; and 118, 74%, 136 – H_2O. The apparent molecular ion at m/z 432 suggests a trihydroxylated derivative of vitamin D_3. The series of diagnostic ions at m/z 271, 253, 136, and 118 show that the seco-steroid nucleus and 3β-hydroxyl group of vitamin D_3 are intact and that the three additional hydroxyl groups are located on the side chain, but their position cannot be assigned from this spectrum.

After partial silylation of 700 ng of compound IV, the mass spectrum shown in Figure 10 was obtained. Major ions, relative intensities, and structural assignments are as follows: m/z 720, 8, M^+ [$(Me_3Si)_4$ derivative]; 648, 21, M^+ [$(Me_3Si)_3$ derivative]; 633, 2, 648 – CH_3; 617, 1, 720 – CH_2OSiMe_3; 558, 3, 648 – $HOSiMe_3$; 543, 3, 558 – CH_3; 501, 7, 720 – $C_3H_5(OSiMe_3)_2$ or 648 – $C_3H_5OHOSiMe_3$; 411, 4, 501 – $HOSiMe_3$; 343, 2, M^+ – side chain; 321, 2, 411 – $HOSiMe_3$; 253, 8, 343 – $HOSiMe_3$; 219, 67, $C_3H_5(OSiMe_3)_2^+$; 208, 52, (A ring + C_6 + $C_7)^+$; 147, 62, $C_3H_5OHOSiMe_3^+$; 131, 79,

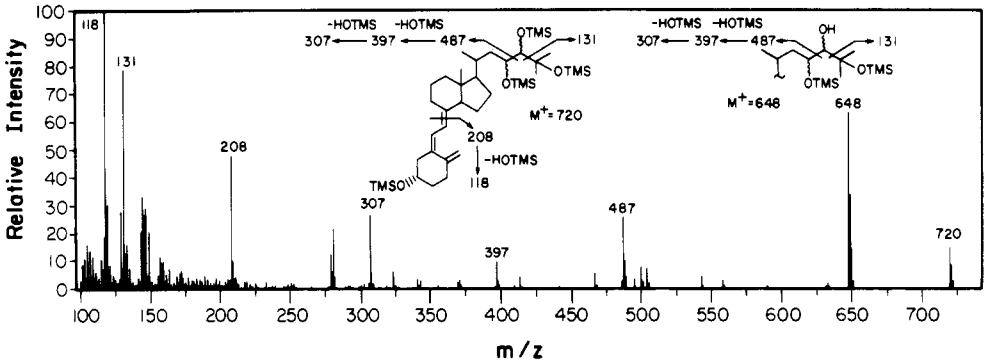

FIGURE 8: Mass spectrum of partially trimethylsilylated 23,24,25-$(OH)_3D_3$. The part structure shown as an inset represents one of the three possible disilylated side-chain structures. The intensities of ions above m/z 260 are enhanced by a factor of 5.

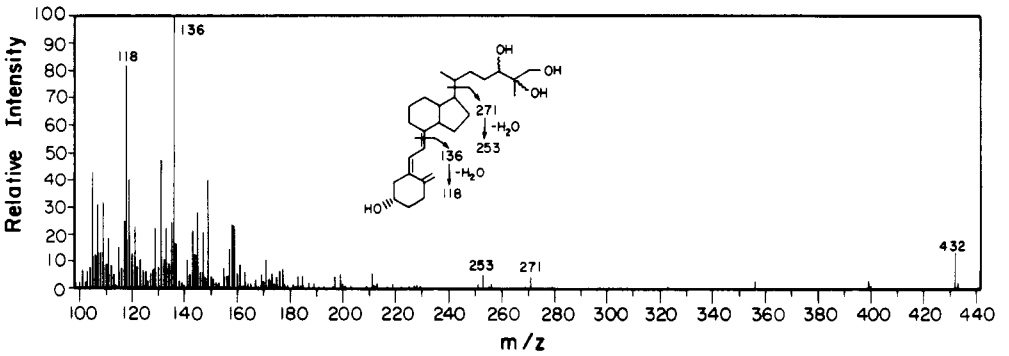

FIGURE 9: Mass spectrum of 24,25,26-$(OH)_3D_3$.

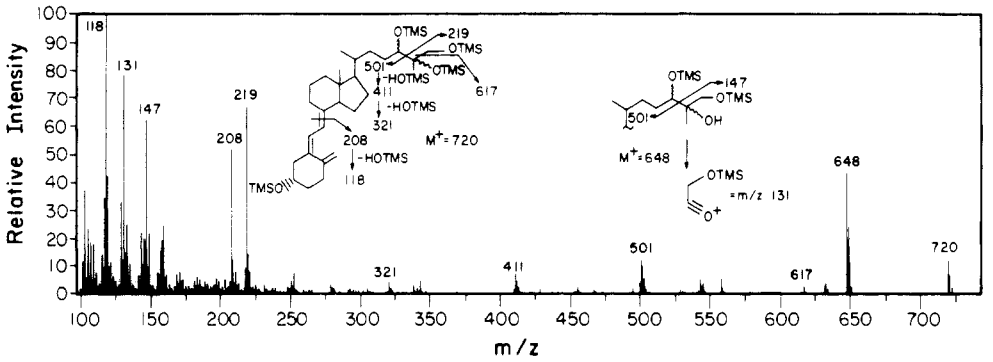

FIGURE 10: Mass spectrum of partially trimethylsilyated 24,25,26-$(OH)_3D_3$. The part structure shown as an inset represents one of the three possible disilylated side-chain structures. The intensities of ions above m/z 260 are enhanced by a factor of 2.

$C_2H_2OOSiMe_3^+$; 118, 100, 208 – $HOSiMe_3$. The molecular ion at m/z 720 is expected for a $(Me_3Si)_4$ derivative of a trihydroxylated vitamin D_3 metabolite, whereas the ion at m/z 648 is the expected molecular ion of the $(Me_3Si)_3$ derivative of the same compound. This result confirms the presence of three side-chain hydroxy groups, and the pattern of ions at m/z 343 (M^+ – side chain) and 253 (343 – $HOSiMe_3$) as well as the diagnostic peaks at m/z 208 and 118 confirms the intact seco-steroid nucleus including a C-3 hydroxy function. The position of two of the side-chain hydroxyl groups at C-25 and C-26 is apparent from the prominent peak at m/z 219 which arises by C-24–C-25 bond cleavage with charge retention as the smaller side-chain fragment (see Figure 10). The same ion occurs in the mass spectra of trimethylsilylated 25,26-$(OH)_2D_3$ and of 25,26-disilylated steroid models. The peak at m/z 147 is interpreted as the corresponding monosilylated fragment contributed by the species of M^+ = 648 (see Figure 10), although the possibility that the peak might represent a siloxane ion $[(CH_3)_3SiO^+{=}Si(CH_2)_2]$ is not excluded. (The prominence of this peak and its absence in the spectra of other silylated derivatives strongly suggest, however, that the former interpretation is correct.) The small peaks at m/z 617 and 545 representing loss of a fragment of mass 103 from the molecular ions at m/z 720 and 648, respectively, and the presence of a prominent peak at m/z 103 ($CH_2OSiMe_3^+$) corroborate a vicinal C-25,26 diol structure, since the analogous fragmentation is observed in the spectrum of 25,26-$(OH)_2D_3$-$(Me_3Si)_3$ ether (Suda et al., 1970). The remaining hydroxy group must be assigned to C-24 by virtue of the prominent peak at m/z 501 (and the fragments at m/z 411 and 321 resulting from successive elimination of $HOSiMe_3$). This peak also results from C-24–C-25 bond cleavage but with charge localization on the larger fragment. Peaks of similar intensity and arising by the analogous C-24–C-25 bond cleavage are observed in the mass spectra of silylated 24,25-$(OH)_2D_2$ (Jones et al., 1980) and 1,24,25-$(OH)_3D_3$ (Holick et al., 1972). This assignment is confirmed by the complete absence of fragments indicative of hydroxy substitution at any other side-chain positions (e.g., ions of m/z 487, 473, and 459 are expected for a C-23, C-22, or C-20 $OSiMe_3$ substituent, respectively; see also preceding discussion of the spectrum of Figure 8). These data then define the structure of metabolite IV as 24,25,26-$(OH)_3D_3$ (Figure 5, IV).

Discussion

The four metabolites identified in this report add to the complexity of the intermediary metabolism of vitamin D_3. All four compounds conform to the general pattern of vitamin D_3 metabolism in that all structural modifications involve positions previously shown to be subject to metabolic attack. Side-chain metabolism of vitamin D_3 to 24- and 25-OH-D_3 and to 23,25-, 24,25-, and 25,26-dihydroxy-D_3 metabolites is well documented (DeLuca, 1980; Wichmann et al., 1981; Tanaka et al., 1981), and examples of further alteration to a C-24-ketone (Takasaki et al., 1981) to C-23 and C-24 carboxylic acids (Esvelt et al., 1979; DeLuca & Schnoes, 1979) or to a 26,23-lactone derivative (Wichmann et al., 1979) have been reported. Likewise, the introduction of an olefinic unsaturation has precedent in the isolation of 24- and 25-dehydro-1α-OH-D_3 from rat bile (Onisko et al., 1980).

The structures presented here, therefore, represent no significant departure from the known reactions of vitamin D metabolism, and the compounds may represent intermediates of normal, but minor, side-chain metabolism pathways. It must be remembered, however, that these compounds were isolated from animals receiving high doses of vitamin D_3. In the normal animal, these metabolites likely represent minor metabolic products of uncertain functional significance. They may well arise by the nonspecific action of the known vitamin D specific hydroxylases or from the action of enzymes of general steroid metabolism. Under conditions of high vitamin D intake, the circulating levels of 24,25-$(OH)_2D_3$ are significantly elevated (Shepard & DeLuca, 1980), and this metabolite may in fact serve as a precursor for all four compounds discussed here, yielding 23-dehydro-25-OH-D_3 and 24-keto-25-OH-D_3 by dehydration and oxidation and the side-chain trihydroxy derivatives by further hydroxylation of C-23 or C-26. Subsequent side-chain degradation to C-23 or C-24 carboxylic acids which would render the compounds water soluble for rapid excretion via the bile is easily envisioned, and the metabolites may thus represent intermediates of a possibly minor pathway of vitamin inactivation and eventual excretion.

It is also quite possible that these metabolites play a role in vitamin D toxicity, the causes of which are poorly understood at present. At high doses of vitamin D or 25-OH-D, circulating levels of 1,25-$(OH)_2D_3$ fall to undetectable levels (Shepard & DeLuca, 1980). It is thus evident that 1,25-$(OH)_2D_3$ cannot be the cause of vitamin D toxicity. It is plausible that metabolites such as 23-dehydro-25-OH-D_3, 24-keto-25-OH-D_3, 23,24,25-$(OH)_3D_3$, 24,25,26-$(OH)_3D_3$, and the previously reported 24-OH-D_3 and 23,25-$(OH)_2D_3$ metabolites (Wichmann et al., 1981; Tanaka et al., 1981) could be involved in vitamin D toxicity. All of these compounds are present at levels comparable to those of certain physiologic vitamin metabolites (0.5–10 ng/mL). It is possible that these metabolites interfere with the normal feedback controls and the regulation of the calcium and phosphate homeostatic system or perhaps possess intrinsic toxicity. The evaluation of these possibilities will require more material and further investigation, but it is clear that under conditions of high vitamin D intake these metabolites are significant elements of the pattern of vitamin D metabolism and their possible

effects should not be ignored.

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Regulation of Protein Synthesis in *Dictyostelium discoideum*: Effects of Starvation and Anoxia on Initiation†

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ABSTRACT: A rapid reduction in the size and amount of total polysomes is one of the earliest observed changes when the cellular slime mold *Dictyostelium discoideum* is starved for a nutrient source. The measured elongation rate of protein synthesis is similar in both starved and vegetative cells and measures approximately 100 and 120 amino acids per min, respectively. Therefore, the reduction in the size and amount of polysomes following starvation is the result of a rapid decrease in the initiation rate of protein synthesis. Accompanying this reduction in the initiation rate is a decrease in the percentage of cellular messenger ribonucleic acid (mRNA) associated with polysomes from 85–90% in fed cells to 65% in starved cells. When the elongation rate in starved cells is reduced by the addition of cycloheximide to cultures, polysomes increase to vegetative levels in size and amount, demonstrating the lack of inactivation of either ribosomes or mRNA. Polysomes also rapidly increase in amount when starved cells are resuspended in growth medium. When axenic cultures containing exponentially growing cells are shifted from rapid shaking to gentle mixing conditions, the concentration of dissolved oxygen decreases by greater than 95% within 10–15 min. After a short period of time of continued gentle mixing, total polysomes disaggregate, and the rate of protein synthesis drops to 20% of the control rate. When cultures are reoxygenated by being returned to rapid shaking conditions or by bubbling air through the medium, polysomes increase in size and amount, and the rate of protein synthesis rapidly returns to control levels. We conclude from these data that the regulation of protein synthesis in *Dictyostelium* is exquisitely sensitive to the cellular environment. In response to starvation and anoxia, these cells rapidly reduce the initiation rate of protein synthesis. Furthermore, when these cells are returned to normal growth conditions, the initiation rate rapidly returns to vegetative levels.

Eukaryotic cells regulate the rate of protein synthesis in response to a wide variety of environmental conditions. As an example, polysomes disaggregate and the initiation rate of protein synthesis drops in many mammalian cells starved for amino acids or glucose (Christman, 1973; Hogan & Korner, 1968; Lee et al., 1971; Van Venrooij et al., 1972). This block in polypeptide chain initiation also leads to the accumulation in the cytoplasm of mRNA[1] in the form of nonpolysomal messenger ribonucleoprotein. When the missing nutrients are added back to cultures, the amount of polysomes and the initiation rate of protein synthesis in these cells rapidly return to vegetative levels. Furthermore, this increase in the rate of protein synthesis does not require new mRNA synthesis and instead appears to be dependent on more efficient translation of mRNA made prior to the starvation event. Mitosis, hy-

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Cl_3CCOOH, trichloroacetic acid; RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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